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Functional Differences in Nucleoside and Nucleobase Transporters Expressed on the Rabbit Corneal Epithelial Cell Line (SIRC) and Isolated Rabbit Cornea

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Soumyajit Majumdar[1], Giridhar S. Tirucherai[1,2], Dhananjay Pal[1], and Ashim K. Mitra[1]

[1]Division of Pharmaceutical Sciences, School of Pharmacy, University of Missouri-Kansas City, 5005 Rockhill Road, Kansas City, MO 64110

[2]Department of Clinical Pharmacology, Quintiles Inc, 10245 Hickman Mills Drive, Kansas City, MO 64137

ABSTRACT

The purpose of this study was to investigate the expression of nucleoside/nucleobase transporters on the Statens Seruminstitut rabbit corneal (SIRC) epithelial cell line and to evaluate SIRC as an in vitro screening tool for delineating the mechanism of corneal permeation of nucleoside analogs. SIRC cells (passages 410-425) were used to study uptake of [^{3}H]thymidine, [^{3}H]adenine, and [^{3}H]ganciclovir. Transport of [^{3}H]adenine and [^{3}H]ganciclovir was studied across isolated rabbit cornea. Uptake and transport studies were performed for 2 minutes and 120 minutes, respectively, at 34°C. Thymidine uptake by SIRC displayed saturable kinetics (K_m = 595.9 ± 80.4μM, and V_{max} = 289.5 ± 17.2 pmol/min/mg protein). Uptake was inhibited by both purine and pyrimidine nucleosides but not by nucleobases. [^{3}H]thymidine uptake was sodium and energy independent but was inhibited by nitrobenzylthioinosine at nanomolar concentrations. Adenine uptake by SIRC consisted of a saturable component (K_m = 14.4 ± 2.3μM, V_{max} = 0.4 ± 0.04 nmol/min/mg protein) and a nonsaturable component. Uptake of adenine was inhibited by purine nucleobases but not by the nucleosides or pyrimidine nucleobases and was independent of sodium, energy, and nitrobenzylthioinosine. [^{3}H]ganciclovir uptake involved a carrier-mediated component and was inhibited by the purine nucleobases but not by the nucleosides or pyrimidine nucleobases. However, transport of [^{3}H]adenine across the isolated rabbit cornea was not inhibited by unlabeled adenine. Further, corneal permeability of ganciclovir across a 100-fold concentration range remained constant, indicating that ganciclovir permeates the cornea primarily by passive diffusion. Nucleoside and nucleobase transporters on rabbit cornea and corneal epithelial cell line, SIRC, are functionally different, undermining the utility of the SIRC cell line as an in vitro screening tool for elucidating the corneal permeation mechanism of nucleoside analogs.

Corresponding Author: Ashim K. Mitra, Division of Pharmaceutical Sciences, School of Pharmacy, University of Missouri-Kansas City, 5005 Rockhill Road, Kansas City, MO 64110; Tel: (816) 235-1615; Fax: (816) 235-5190; Email: mitraa@umkc.edu.

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INTRODUCTION

Corneal epithelial and stromal keratitis, caused by herpes simplex virus type 1 (HSV-1), is the leading cause of blindness in the United States.[1] Antiviral nucleoside analogs such as trifluridine (TFT) and idoxuridine (IDU), and acyclic nucleoside analogs acyclovir (ACV) and ganciclovir (GCV), have been applied topically to treat such infections.[2-4] However, for topically applied drugs to be effective, the compounds need to penetrate the cornea. The corneal epithelium consists of 5 to 6 layers of columnar epithelial cells that form tight junctions,[5] limiting paracellular diffusion. Since a large number of naturally occurring nucleoside and nucleoside analogs are hydrophilic,[6] transcellular diffusion is also low, thereby limiting the effectiveness of these antiviral agents. In the absence of specialized transport systems, the passage of hydrophilic molecules across the corneal epithelium is thus limited.

In mammalian cells, transcellular flux of nucleosides and nucleobases has been shown to be mediated by specific nucleoside and nucleobase transporters expressed on the cell membranes.[7,8] These transporters are essential for the cellular salvage of nucleosides and nucleobases for nucleotide and nucleic acid synthesis. Nucleoside transporters are classified into 2 categories based on their sodium dependency: sodium-independent equilibrative nucleoside transporters (facilitated diffusion) and concentrative

(sodium dependent) nucleoside transporters.[7-12] Equilibrative nucleoside transporters are further classified into 2 subtypes on the basis of their sensitivity to nitrobenzylthioinosine (NBT). Equilibrative sensitive type, "es," is inhibited by nanomolar concentrations of NBT (K_i 0.1-1nM), whereas the equilibrative insensitive type, "ei," is inhibited by micromolar concentrations of NBT.[7-9] Both the "es" and the "ei" types of equilibrative transporters exhibit broad substrate specificities for the purine and pyrimidine nucleosides.

Sodium-dependent concentrative systems comprise 5 subtypes, N1 to N5,[7-9,13-16] defined on the basis of substrate selectivity. N1 is selective for the purine nucleosides and uridine, N2 is selective for the pyrimidine nucleosides and adenosine, N3 has broad specificity for both purine and pyrimidine nucleosides, and N4 is pyrimidine selective but also transports adenosine and guanosine but not inosine. The N5 system is NBT sensitive and prefers guanosine as the substrate.[9]

Nucleobase transporters, on the other hand, are not so clearly defined. These transporters have been reported to exhibit varying substrate specificities with respect to nucleobases as well as nucleosides.[8,17-22] Moreover, both sodium-dependent and sodium-independent nucleobase transporters are expressed on mammalian cells.

So far the cornea remains fairly unexplored with respect to the expression of nucleoside/nucleobase transporters. Recently, we reported for the first time that the rabbit cornea expresses the N3 type sodium-dependent concentrative nucleoside transporter.[23] We also determined that 2 therapeutically effective anti-HSV agents, ACV and IDU, were not substrates for this transporter. These antiviral nucleoside analogs primarily penetrate the cornea by passive diffusion and thus exhibit limited penetration into the deeper layers of the cornea and the aqueous humor. Nucleoside analogs targeted toward the nucleoside transporter expressed on the cornea represent an exciting and clinically relevant strategy to enhance drug concentrations in the deeper corneal layers as well as in the anterior chamber of the eye. However, drug screening for substrates would require use of a large number of corneas. An objective of this research was to investigate whether the Statens Seruminstitut rabbit corneal (SIRC) cell line can serve as an effective in vitro screening tool in identifying nucleoside drugs with affinity for the nucleoside transporter expressed on the cornea. The SIRC cell line was originally derived from the cornea of a normal hare[24] and has been widely used as an in vitro model to assess corneal transport as well as immunological and toxicological responses.[25-31] However, contradictory reports exist about the epithelial origin of the cell line. Some investigators refer to the cell line as fibroblastic,[24] whereas others claim that it is of epithelial origin.[25-27] Earlier reports from our laboratory have shown that these cells indeed display epithelial morphology.[31] Several studies reported good correlation between permeability coefficients across SIRC cell line and intact rabbit cornea.[25,26,31] Recently, we reported the expression of a large neutral amino acid transporter on the SIRC cell line as well as on the rabbit cornea and the utility of this cell line in screening for prodrugs targeted toward this transporter.[32,33] These studies, reporting good correlation between uptake by the SIRC cell line and transport across the isolated corneal tissue, suggested that the cell line might serve as a good in vitro model for the rabbit cornea. SIRC is an immortalized cell line and can be propagated several times, as opposed to primary cultures, rendering it more useful as a high-throughput screening tool. Our objective was to determine whether nucleoside/nucleobase transporters are expressed on both the SIRC cell line and the isolated rabbit cornea, with a view toward validating the cell line for further studies. Since our earlier observations indicated that thymidine was transported across the rabbit cornea by a sodium-dependent nucleoside transporter,[23] thymidine was selected as the model nucleoside. GCV was chosen as the model nucleoside drug and adenine as the model nucleobase, since the adenine transporter has been reported to be involved in the transport of GCV.[34,35]

MATERIALS AND METHODS

Materials

[^{3}H]GCV (9.5 Ci/mmol) and [^{3}H]adenine (14 Ci/mmol) were procured from Moravek Biochemicals (Brea, CA). [^{3}H]thymidine (26 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). [^{14}C]mannitol (231 mCi/mmol) was obtained from ICN Biomedicals Inc (Irvine, CA). GCV was a gift from Hoffman La Roche (Nutley, NJ). Minimum essential medium (MEM) and 10% fetal bovine serum (FBS) were purchased from Gibcoryl (Grand Island, NY). Culture plates were obtained from Costar (Corning, NY). All other chemicals were purchased from Sigma Chemical Company (St Louis, MO) and used without further purification.

Cell Culture

SIRC cells were obtained from American Type Culture Collection, Manassas, VA, at passage 400 and used between passages 410 and 425. Culture media consisted of

MEM containing 10% FBS, lactalbumin, hydroxyl ethyl piperazine ethane sulfonicacid (HEPES), sodium bicarbonate, penicillin (100 µg/mL), and streptomycin (100 µg/mL). The medium was replaced every other day. Cells were maintained at 37°C, in a humidified atmosphere of 5% CO_2 and 90% relative humidity. For uptake studies, cells were plated at a density of 500 000 cells/well on 12-well culture plates and incubated at 37°C.

Uptake Studies

Uptake studies were conducted using confluent cultures 10 to 12 days postseeding. The medium was aspirated, and cells were washed 3 times with Dulbecco's phosphate-buffered saline (DPBS) pH 7.4 (130mM NaCl, 7.5mM Na_2HPO_4, 1.5mM KH_2PO_4, 0.5mM $MgSO_4$, 1mM $CaCl_2$, 0.03mM KCl, and 5mM glucose) and equilibrated for 1 hour with the buffer. Uptake was carried out for 2 minutes at 34°C (physiological temperature of the rabbit cornea). To determine the sodium dependence of the uptake process, NaCl was replaced with choline chloride, and Na_2HPO_4 was replaced with K_2HPO_4, in equimolar quantities.

Uptake study was initiated by the addition of 2 mL of drug solution (in DPBS) and was terminated after 2 minutes by the addition of ice-cold stop solution (0.52 g/L HEPES and 15.64 g/L KCl). After 3 washings with ice-cold stop solution, cells were lysed overnight, at room temperature, using 1 mL of Triton-X solution (0.1% vol/vol) in 0.3N NaOH. Aliquots (500 µL) from each well were then transferred to scintillation vials containing 5 mL of scintillation cocktail (Fisher Scientific, Fairlawn, NJ). Samples were analyzed with a scintillation counter (Model LS-6500, Beckman Coulter (Fullerton, CA), and rate of uptake was normalized to protein content of each well. Cell lysate protein content was measured by the method of Bradford[36] using bovine serum albumin as the standard (Bio-Rad protein estimation kit, Hercules, CA). Nonspecific binding was corrected by carrying out the uptake at 4°C.

Animals

Adult male New Zealand albino rabbits weighing 2 to 2.5 kgs were obtained from Myrtle's Rabbitry (Thompson station, TN). Experiments involving rabbits conformed to the tenets of the Association for Research in Vision and Ophthalmology statement on animal use in ophthalmic and vision research.

Corneal Transport Studies

Permeation of radiolabeled nucleosides ([^{3}H]adenine) and nucleoside analogs ([^{3}H]GCV) across isolated rabbit cornea was studied using side-by-side diffusion cells (type-VSC-1, Crown Glass Company Inc, (Somerville, NJ). Corneal membranes isolated from male New Zealand albino rabbit eyes were employed for these studies. Animals were euthanized by an overdose of pentobarbital through the marginal ear vein. Eyes were proptosed, carefully enucleated, and washed with DPBS (pH 7.4) to remove any trace of blood. After a small incision to the sclera, vitreous humor was aspirated using a 1-mL tuberculin syringe. The cornea was carefully excised, leaving some adhered scleral portion. The scleral part attached to the cornea was used to hold the tissue in place between the diffusion half-cells during transport experiments. The lens was removed, and the iris-ciliary body was separated from the cornea using forceps. The cornea was washed immediately with DPBS and mounted on the side-by-side diffusion chambers. The temperature was maintained at 34°C by circulating water through the jacketed chambers of the diffusion apparatus. Both chambers were placed on automated drive consoles that aided in continuous stirring of the donor and receiver cell solutions.

Permeant solutions (3 mL) were added on the epithelial side of the cornea (donor chamber). In the receiver chamber, 3.2 mL of DPBS was added. The receiver chamber was kept at a slightly higher volume than the donor chamber such that the hydrostatic pressure difference maintained the natural curvature of the cornea throughout the experiment. The contents in both chambers were stirred continuously using magnetic stir bars. Total duration of the permeation experiment was 2 hours. Donor solution consisted of 0.5 µCi/mL of [^{3}H]adenine or [^{3}H]GCV, in the presence or absence of various nucleosides and nucleoside analogs. Samples of 100 µL were withdrawn from the receiver chamber at selected time points and placed into scintillation vials. Sample volumes were immediately replaced with equal volumes of DPBS. A 5-mL scintillation cocktail was then added to each sample and analyzed in the scintillation counter.

Control experiments using [^{14}C]mannitol (0.3 µCi/mL or 1.29 nmol/mL), a paracellular marker, were carried out to evaluate the corneal integrity over the time course of the experiment.

Data Treatment

Uptake data for GCV and adenine were fitted to the modified Michaelis-Menten equation shown in Equation 1:

$$V_T = \frac{V_{max} \times C}{K_m + C} + k_d \times C \quad (1)$$

Equation 1 takes into account a carrier-mediated process (as described by the classical Michaelis-Menten equation) and a nonsaturable passive diffusion process.

V_T represents the total rate of uptake, V_{max} is the maximum rate of uptake for the carrier-mediated process, K_m denotes the concentration at half-saturation (Michaelis-Menten constant), C represents substrate concentration, and k_d is the rate constant for the nonsaturable diffusional component.

Data were analyzed according to Equation 1 with a nonlinear least squares regression analysis program (Kaleida Graph V3.09, Synergy Software, PA).

In Equation 1, $k_d \times C$ represents the nonsaturable component, whereas the saturable component of total uptake is represented by $(V_{max} \times C) / (K_m + C)$. The kinetic parameters, calculated using Kaleida Graph, were substituted into the equation to determine the contribution of the saturable and nonsaturable components.

Cumulative amounts of [^{3}H]GCV and [^{3}H]adenine appearing in the receptor phase during a transport experiment were plotted as a function of time to determine the permeability coefficient. Linear regression of [^{3}H]GCV and [^{3}H]adenine amounts versus time plots yielded the rate of transport across the cornea (dM/dt). The rate divided by the cross-sectional area available for diffusion (A) generated the steady state flux:

$$\text{Flux} = (dM/dt)/A \quad (2)$$

Corneal permeability was determined by normalizing the steady state flux to the donor concentration (C_d) of the drug:

$$\text{Permeability} = \text{Flux}/C_d \quad (3)$$

Statistical Analysis

All experiments were conducted at least in triplicate, and results are expressed as mean ± SD. Student t test was used for statistical analysis, and $P < .05$ was considered to be statistically significant.

RESULTS

Studies Using SIRC Cell Line

Concentration-Dependent Uptake of Adenine and Thymidine

To determine concentration dependency, uptake studies were conducted by spiking 0.5 μCi/mL of [^{3}H]adenine or [^{3}H]thymidine to various concentrations of unlabeled adenine (0.1-500μM) or thymidine (0.0025-5mM), respectively. Uptake of adenine was found to consist of a saturable component (carrier-mediated process) and a nonsaturable component (**Figure 1**). Values for the kinetic constants K_m and V_{max} for adenine uptake were calculated to be 14.4 ± 2.3μM and 0.4 ± 0.04 nmol/min/mg protein, respectively. k_d had a value of 0.45 ± 0.02 μL/min/mg protein. Saturable and nonsaturable components of adenine uptake process (**Figure 1**) were determined by substituting the values of the kinetic constants into Equation 1.

[^{3}H]thymidine uptake also exhibited saturable kinetics (**Figure 2**) and was analyzed by the Michaelis-Menten equation. Values for the kinetic constants K_m and V_{max} were calculated to be 595.9 ± 80.4μM and 289.5 ± 17.2 pmol/min/mg protein, respectively.

Effect of Nucleosides, Nucleobases, and Nucleoside Analogs on Uptake of [^{3}H]Adenine

Uptake studies were carried out with 0.5 μCi (35.7 pmol/mL) of [^{3}H]adenine in the presence and absence of various nucleosides (adenosine, guanosine, and thymidine), nucleobases (unlabeled adenine, guanine, hypoxanthine, thymine, and uracil) and antiviral nucleoside analogs (ACV, GCV, and IDU), as inhibitors of adenine uptake, to examine the substrate specificity of the transporter (**Table 1**). All inhibitors were at 1mM concentrations. Unlabeled adenine drastically inhibited (98% ± 0.06%) SIRC uptake of [^{3}H]adenine. Substantial inhibition was also achieved with hypoxanthine (66% ± 1.05%) and guanine (34% ± 3.7%). However, the pyrimidine nucleobases thymine and uracil did not affect adenine uptake. Neither the nucleosides nor the nucleoside analogs produced any significant inhibition of [^{3}H]adenine uptake (**Table 1**).

Effect of Nucleosides, Nucleobases, and Nucleoside Analogs on Uptake of [^{3}H]Thymidine

Uptake studies were carried out with [^{3}H]thymidine, 0.5 μCi/mL (19.2 pmol/mL), in the presence and absence of

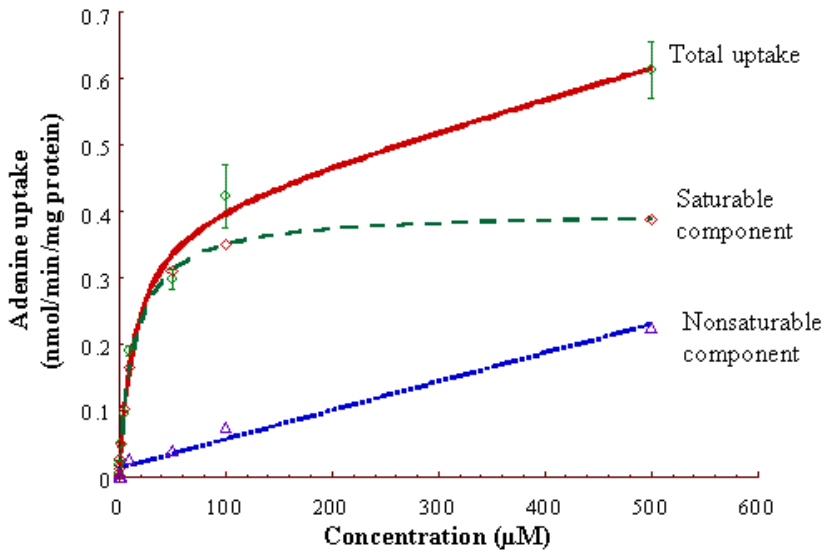

Figure 1. Concentration-dependent uptake of adenine by SIRC cells. Studies were conducted by spiking 0.5 µCi/mL of [^{3}H]adenine to various concentrations of unlabeled adenine. Saturable and nonsaturable components were determined by substituting the values of the kinetic constants into Equation 1. Data represent mean ± SD (n = 3).

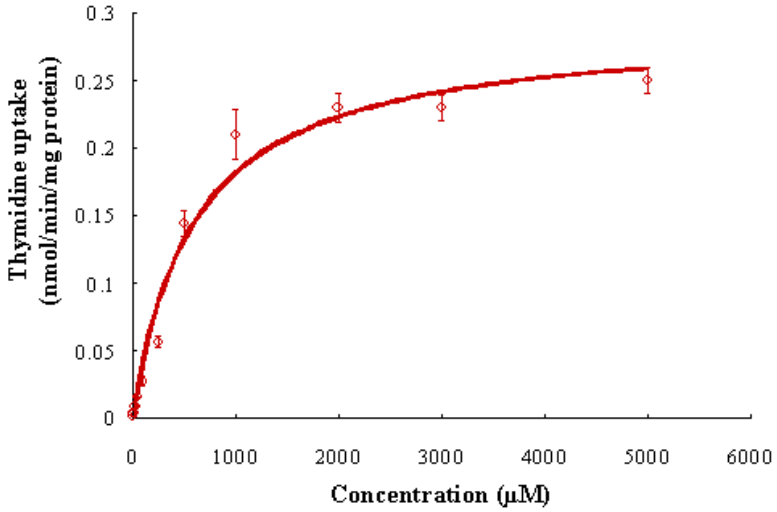

Figure 2. Concentration-dependent uptake of thymidine by SIRC cells. Studies were conducted by spiking 0.5 µCi/mL of [^{3}H]thymidine to various concentrations of unlabeled thymidine. Data represent mean ± SD (n = 3).

various nucleosides, nucleobases, and antiviral nucleoside analogs to examine the substrate specificity of the thymidine transporter. Uptake was significantly decreased in the presence of both purine (adenosine, guanosine, and inosine) and pyrimidine (thymidine and uridine) nucleosides (**Table 2**). The transport system was most sensitive to thymidine and least sensitive to guanosine. **Table 2** also shows the effect of purine (adenine and guanine) and pyrimidine (uracil) nucleobases on the uptake of [^{3}H]thymidine. Uptake was decreased by 31% to 95% of the control in the presence of nucleosides, whereas the nucleobases did not produce any significant inhibition, indicating that the nucleosides are preferred substrates for this transporter.

The acyclic nucleoside analogs ACV and GCV did not exhibit a significant inhibitory effect on [^{3}H]thymidine uptake (**Table 2**). However, nucleoside analogs TFT, IDU, and azidothymidine (AZT) produced a dramatic decrease in [^{3}H]thymidine uptake.

Table 1. Effect of Nucleobases, Nucleosides, and Nucleoside Analogs on the Uptake of [^{3}H]Adenine (0.5 μCi/mL) by SIRC Cells*

Inhibitors	Uptake of [^{3}H]Adenine as a % of Control	SD
Effect of nucleobases		
Adenine 1mM	2.7	0.1*
Guanine 1mM	65.6	3.7*
Hypoxanthine 1mM	33.6	1.1*
Thymine 1mM	84.1	7.5
Uracil 1mM	83.5	8.2
Effect of nucleosides		
Adenosine 1mM	88.2	8.2
Guanosine 1mM	85.6	7.6
Thymidine 1mM	105.4	5.4
Effect of nucleoside analogs		
Acyclovir 1mM	90.2	3.5
Ganciclovir 1mM	92.5	5.8
Idoxuridine 1mM	107.1	5.8

*$P < .05$.

Sodium and Energy Dependence of [^{3}H]Thymidine and [^{3}H]Adenine Uptake

Uptake of 0.5 μCi/mL of [^{3}H]thymidine and [^{3}H]adenine was carried out in the presence and absence of sodium to determine whether the process was sodium dependent. Absence of sodium in the uptake medium did not have any significant effect on the uptake of [^{3}H]thymidine or [^{3}H]adenine, indicating that the uptake process was sodium independent (**Figure 3**). Inhibitors for Na^+/ K^+-ATPase (ouabain 1mM) and metabolic inhibitors (sodium azide 1mM) were used to further characterize the transport system. Significant differences were not observed in uptake of [^{3}H]thymidine or [^{3}H]adenine between control cells and cells preincubated with sodium azide or ouabain, indicating that uptake was sodium and energy independent (**Figure 3**).

Effect of NBT on [^{3}H]Thymidine and [^{3}H]Adenine Uptake

Sodium-independent nucleoside transport systems are classified into "es" and "ei" types based on their sensitivity to NBT. The "es" type is extremely sensitive to NBT even at nanomolar concentrations (K_i 0.1-10 nM), whereas the "ei" transporter is not inhibited even at 1μM concentrations of NBT. To distinguish between the 2 transporter types on SIRC cells, the nucleoside transporter showing sensitivity to 50nM NBT was classified as the "es" type, and the nucleoside transporter not sensitive to 50nM NBT but sensitive to 250μM NBT was classified as the "ei" type. Stock solutions of NBT were prepared in dimethyl sulfoxide (DMSO). However, the final concentration of DMSO in the uptake medium was less than 2.5%. In these experiments, cells were preincubated with NBT for 15 minutes before initiating uptake. Control studies indicated that 2.5% DMSO did not have any effect on uptake of [^{3}H]adenine or [^{3}H]thymidine by SIRC (data not shown). **Figure 4** shows the inhibitory effect of NBT on [^{3}H]thymidine uptake. The 50nM NBT inhibited the uptake of [^{3}H]thymidine by 80% ("es" component). No further inhibition was noted up to 5μM of NBT (**Figure 4**). However, in the presence of 0.25mM NBT, uptake of [^{3}H]thymidine was decreased by a further 10% ("ei" component) (**Figure 4** insert). These data clearly indicate that the SIRC cell line expresses the "es" type of nucleoside transporter as the major component, with the possible presence of the "ei" type as a minor component. In comparison, NBT did not

Table 2. Effect of Nucleosides, Nucleobases, and Nucleoside Analogs on the Uptake of [^{3}H]Thymidine (0.5 μCi/mL) by SIRC Cells

Inhibitors	Uptake of [^{3}H]Thymidine as a % of Control	SD
Effect of nucleosides		
Thymidine 1mM	5.2	0.4
Uridine 1mM	31.7	6.2
Adenosine 1mM	15.5	3.6
Guanosine 1mM	68.5	4.9
Inosine 1mM	35.4	1.4
Effect of nucleobases		
Guanine 1mM	89.7	8.5
Adenine 1mM	92.8	4.5
Uracil 1mM	94.7	9.3
Effect of nucleoside analogs		
Acyclovir 1mM	93.4	4.5
Ganciclovir 1mM	95.6	6.8
Azidothymidine 1mM	5.1	0.3
Trifluridine 1mM	4.2	0.6
Idoxuridine 1mM	5.4	0.2

*$P < .05$.

show any statistically significant effect on adenine uptake (data not shown).

Concentration-Dependent Uptake of [^{3}H]GCV

Uptake data of GCV by the SIRC cell line indicated the involvement of 2 processes: a carrier-mediated (saturable) component at low concentrations, and an apparent nonsaturable component at higher concentrations (**Figure 5**). Studies were conducted by spiking 0.5 μCi/mL of [^{3}H]GCV to various concentrations of unlabeled GCV (0.05-5mM). Data were modeled using a modified Michaelis-Menten equation according to Equation 1, and the kinetic parameters of GCV uptake were calculated. The values for the kinetic constants K_m and V_{max} were calculated to be 1.29 ± 0.18mM and 1.64 ± 0.36 nmol/min/mg protein, respectively. k_d had a value of 0.6506 ± 0.063 μL/min/mg protein. Saturable and nonsaturable components of GCV uptake by SIRC cells (**Figure 5**) were determined by substituting the values of the kinetic constants into Equation 1.

Inhibitory Effect of Nucleosides and Nucleobases on Uptake of [^{3}H]GCV

Table 3 shows the uptake of 0.5 μCi/mL (52.6 pmol/mL) [^{3}H]GCV in the absence (control) and presence of various nucleobases (adenine, guanine, thymine, and uracil) or nucleosides (adenosine, guanosine, and thymidine), respectively. While adenine produced significant inhibition (78% ± 1.1%) of [^{3}H]GCV uptake by SIRC cells, the other nucleosides or nucleobases did not produce any substantial inhibition. The calculated value of the passive component of GCV uptake, obtained by substituting the kinetic parameters obtained from **Figure 5** into Equation 1, at this concentration is 33.8%. Thus, mathematically, 1mM adenine should have produced 67% inhibition of GCV transport. The experimental and calculated values are therefore close.

Studies With Intact Rabbit Cornea

Transport of [^{3}H]Adenine Across Isolated Rabbit Cornea

Rate of [^{3}H]adenine (0.5 μCi/mL) transport was not affected in the presence of unlabeled adenine, indicating

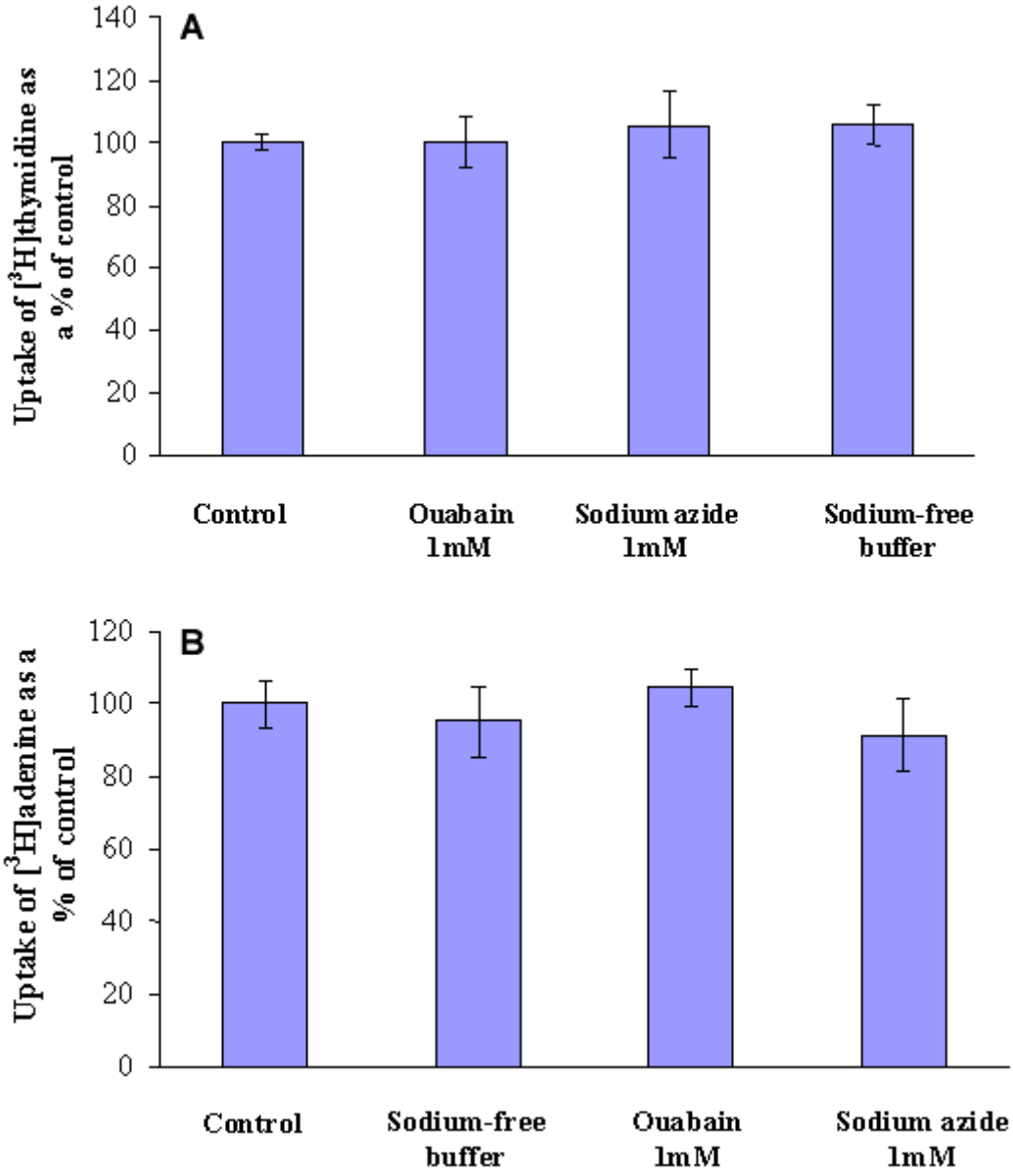

Figure 3. Sodium and energy dependence of (A) [^{3}H]thymidine uptake by SIRC cells; and (B) [^{3}H]adenine uptake by SIRC cells. Data represent mean ± SD (n = 3).

that permeation of adenine across the rabbit cornea probably did not involve any carrier-mediated process (**Figure 6**).

Transport of [^{3}H]Adenine Across Isolated Rabbit Cornea

HPMC gels were prepared at 3% wt/wt while CMC gels were prepared at 1% wt/wt. To potentiate the effect of iontophoresis, terpene enhancers were added to the drug vehicle at a 2% concentration. The cumulative amount of BH permeated across hairless mouse skin using iontophoresis at a current density of 0.1 mA/cm^2 from HPMC and CMC gels was compared to that of the ethanol:water solution. The permeation profile of BH from the gel formulations showed no significant difference from that of the ethanol:water solution (**Figure 6**). The BH flux from HPMC was 55.59 ± 11.18 µg/(h.cm^2), from the ethanol:water solution was 31.42 ± 7.30 µg/(h.cm^2), and from CMC was 27.19 ± 4.15 µg/(h.cm^2).

Terpene enhancers were incorporated to enhance the effect of iontophoresis and to decrease the load of each enhancement technique. **Figure 7** and **Figure 8** show the effect of terpene enhancers on the flux of BH upon the application of 1 mL gel/cell. Terpene enhancers were superior to iontophoresis. In general, terpene enhancers

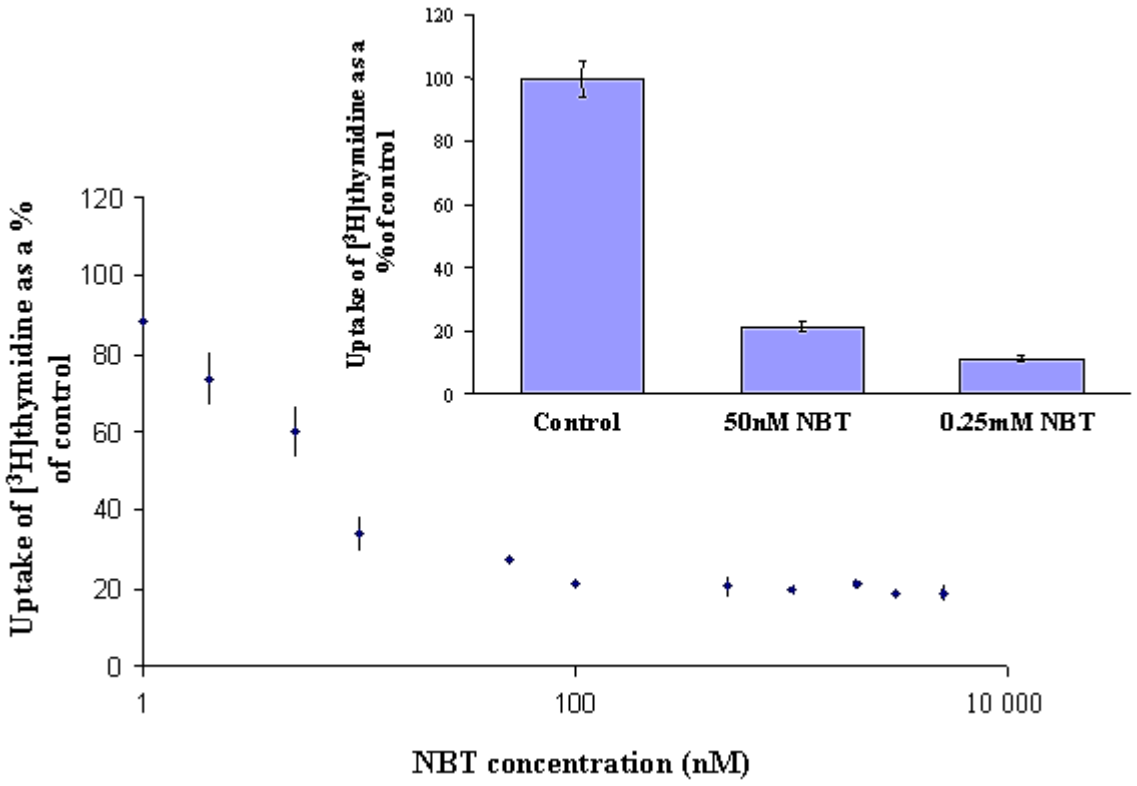

Figure 4. Dose-dependent NBT inhibition (1-5μM) of [^{3}H]thymidine uptake. Insert: [^{3}H]thymidine uptake in the absence (control) and presence of 50nM or 0.25mM NBT. Data represent mean ± SD.

increased the flux of BH by more than 200-fold relative to a 15-fold increase using iontophoresis alone. However, compared with cineole and terpineol, menthol showed the highest activity. Menthol, cineole, and terpineol increased flux values of BH by 300-, 148-, and 235-fold using HPMC gels and 204-, 140-, and 198-fold using CMC compared to that of the control (HPMC and CMC gels), respectively. However, the use of terpene enhancers in combination with iontophoresis resulted in a synergistic effect on the flux of BH from CMC gel. BH flux increased from 27.19 ± 4.15 and 347.10 ± 14.96 when iontophoresis or menthol were used alone to 546.84 ± 40.54 μg/(h.cm^2) when both were combined. This is a synergistic effect. In contrast, the flux of BH from HPMC gels was increased from 55.59 ± 11.18 and 523.10 ± 80.34 using iontophoresis and menthol alone to 637.81 ± 92.88 μg/ (h.cm^2) when menthol was combined with iontophoresis. This marginal increase is statistically insignificant and indicates that no synergy occurred under these conditions.

Transport of GCV Across Isolated Rabbit Cornea

Permeability studies were carried out at various concentrations of GCV (0.02, 0.2, and 2.0mM). Solutions were prepared by spiking 0.5 μCi/mL of the radiolabeled species to the unlabeled drug solutions. The amount of radiolabeled species appearing on the donor side was analyzed, and total GCV transported was calculated. Permeability values were determined using Equation 3. As depicted in **Figure 7**, permeability values for GCV did not show any statistically significant difference over the concentration range studied.

Moreover, [^{3}H]GCV transport across the cornea was not affected in the presence of 1mM adenine (**Figure 8**).

DISCUSSION

Our main objective in this study was to investigate whether the SIRC cell line could be employed as an in vitro tool for screening nucleoside analogs developed for topical administration.

Uptake of thymidine by the SIRC cells exhibited saturable kinetics (**Figure 2**), indicating the expression of a nucleoside and/or nucleobase transporter by the SIRC cells.

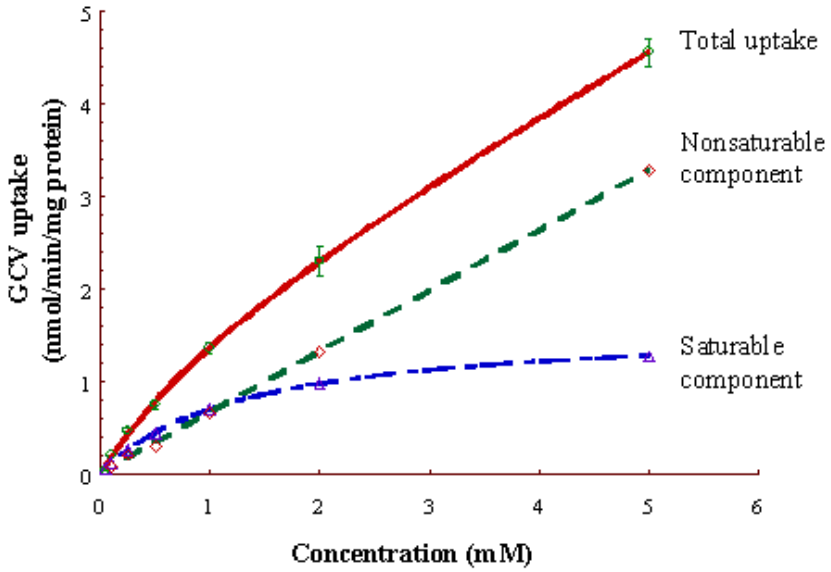

Figure 5. Concentration-dependent uptake of GCV by SIRC cells. Studies were conducted by spiking [^{3}H]GCV (0.5 µCi/mL) to various concentrations of unlabeled GCV. Saturable and nonsaturable components were determined by substituting the values of the kinetic constants into Equation 1. Data represent mean ± SD (n = 3).

Table 3. Effect of Nucleobases and Nucleosides on the Uptake of 0.5 µCi/mL of [^{3}H]GCV by SIRC Cells

Inhibitors	Uptake of [^{3}H]GCV as a % of Control	SD
Effect of nucleobases		
Adenine 1mM	22.4	1.1*
Guanine 1mM	76.0	6.0*
Thymine 1mM	102.4	6.7
Uracil 1mM	88.7	3.8
Effect of nucleosides		
Adenosine 1mM	84.9	7.8
Guanosine 1mM	87.8	9.6
Thymidine 1mM	92.2	5.2

*$P < .05$.

[^{3}H]thymidine uptake was inhibited by both purine and pyrimidine nucleosides but was not affected by the nucleobases (**Table 2**). Moreover, uptake of [^{3}H]thymidine was independent of energy and sodium (**Figure 3**) but was sensitive to nanomolar concentrations of NBT (**Figure 4**). Broad substrate specificity for nucleosides, sodium independence, and sensitivity to nanomolar concentrations of NBT suggest that the transporter expressed on the SIRC cells is of the "es" type equilibrative nucleoside transporter.

Adenine uptake by SIRC cells exhibited a saturable component as well as a passive diffusion component (**Figure 1**). [^{3}H]adenine uptake was inhibited by purine nucleobases (unlabeled adenine, guanine, and hypoxanthine) but not by pyrimidine nucleobases (thymine and uracil) (**Table 1**). Nucleosides did not have any significant inhibitory effect on [^{3}H]adenine uptake (**Table 1**). The uptake

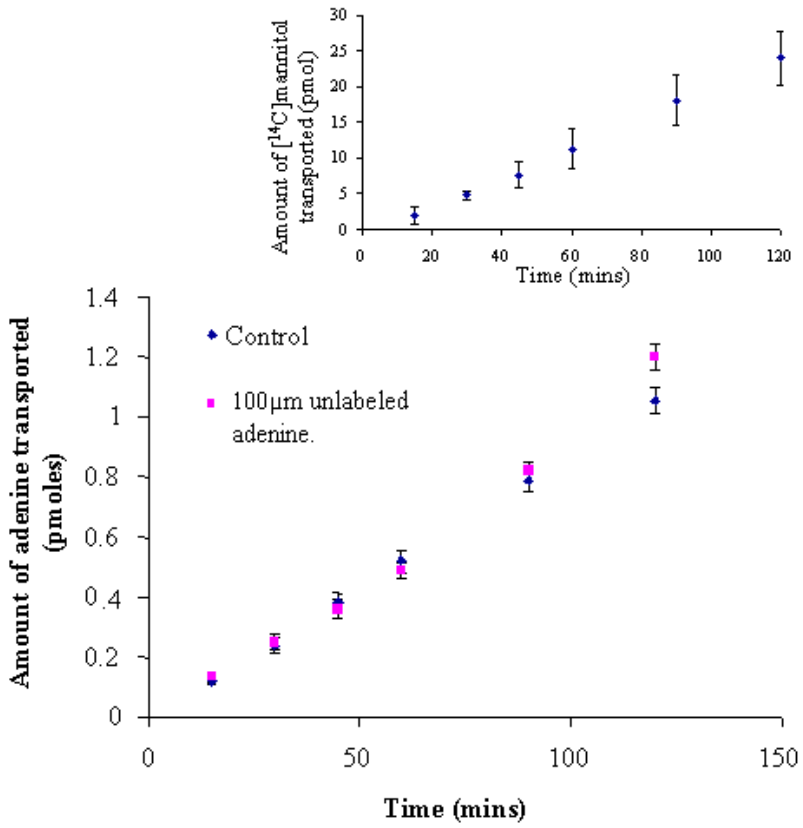

Figure 6. Transport of [^{3}H]adenine (0.5 µCi/mL) across the isolated rabbit cornea in the absence (control) or presence of 100µM unlabeled adenine (n = 3). Insert: [^{14}C]mannitol transport, across isolated cornea, as a function of time (n = 6). Data represent mean ± SD.

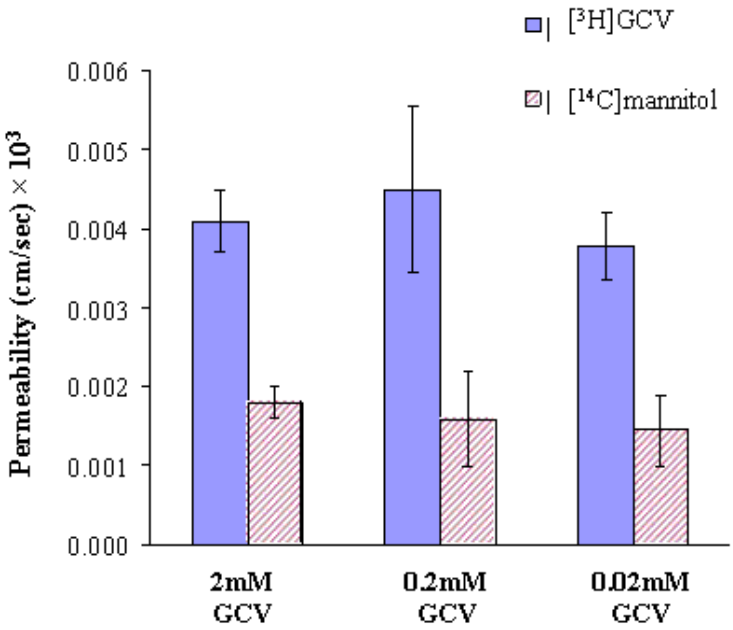

Figure 7. Permeability of [^{3}H]GCV (0.5 µCi/mL) and [^{14}C]mannitol (0.3 µCi/mL) across excised rabbit cornea, in the presence of 2mM, 0.2mM, and 0.02mM unlabeled GCV. Data represent mean ± SD (n = 3).

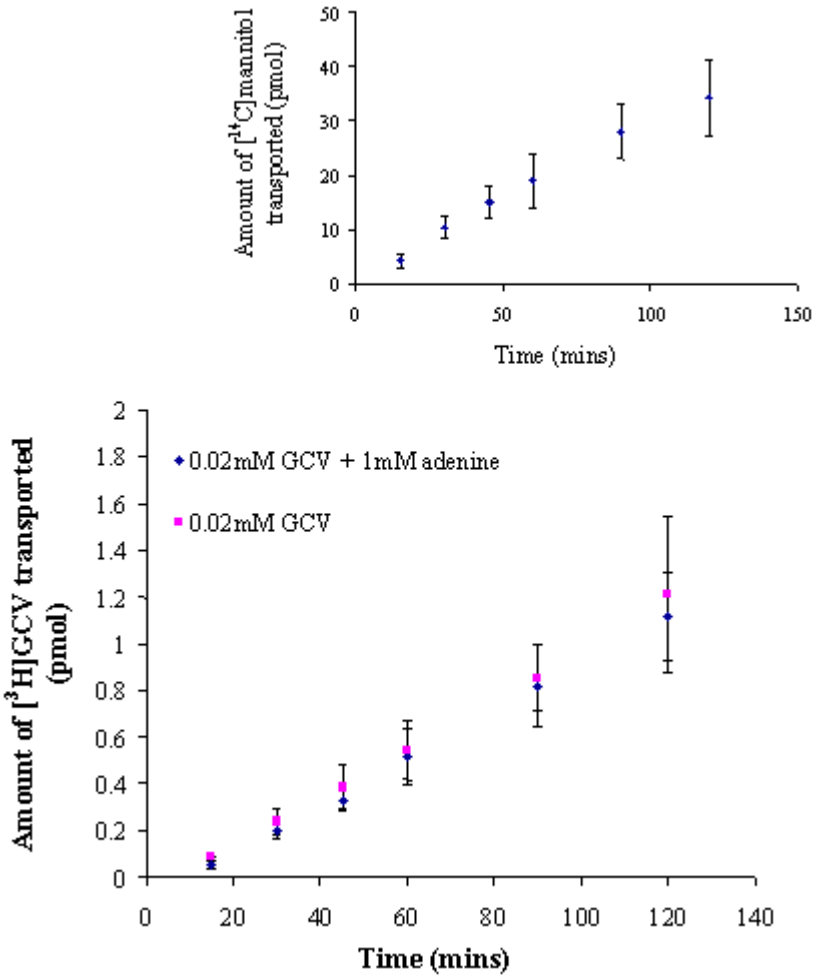

Figure 8. Effect of adenine on transport of [^{3}H]GCV across the isolated rabbit cornea. Studies were conducted by spiking [^{3}H]GCV (0.5 µCi/mL) to solutions containing 0.02mM unlabeled GCV (control) or 0.02mM unlabeled GCV and 1mM adenine (n = 3). Insert: [^{14}C]mannitol transport as a function of time (n = 4). Data represent mean ± SD.

process was independent of sodium (**Figure 3**) and was insensitive to NBT. These results indicate that the SIRC cells also express a purine-specific nucleobase transporter, along with an "es" type nucleoside transporter.

However, these observations are in sharp contrast to the observations on the intact cornea with respect to nucleoside/nucleobase transporters.[23] Thymidine transport across the cornea involves a sodium-dependent N3 type nucleoside transporter (K_m = 14.9 ± 9.7µM). On the other hand, thymidine uptake by the SIRC cells involves an equilibrative ("es") nucleoside transporter (K_m = 595.9 ± 80.4µM). Uptake of adenine by the SIRC cell line appears to be mediated by a purine-specific nucleobase transporter, whereas corneal transport studies suggest that permeation of adenine (**Figure 6**) and thymine (data not shown) does not involve any nucleobase transport system. The 2 models thus do not seem to correlate with respect to expression of nucleoside/nucleobase carrier systems.

Uptake of GCV by SIRC cells was found to involve a minor saturable component (**Figure 5**). Inhibition studies with various nucleosides and nucleobases indicated that GCV uptake was significantly inhibited by adenine, and to some extent by guanine, but not by the other nucleosides and nucleobases (**Table 3**), suggesting that the drug probably shares the adenine transporter. Earlier reports have also shown GCV to be a substrate for nucleobase transporters.[34,35] Interestingly, GCV did not produce any significant inhibition in the uptake of [^{3}H]adenine by the SIRC cells. These data may be explained by the fact that an almost 90-fold difference exists between the K_m of adenine (14.4 ± 2.3µM) and that of GCV (1.29 ± 0.18mM).

GCV at 1mM would be occupying less than half the available capacity of the nucleoside transporter, while 35.7nM [^{3}H]adenine would occupy only a very small fraction. Thus, at these concentrations the transporter is not saturated, and hence GCV is unable to inhibit uptake of [^{3}H]adenine. On the other hand, when 1mM adenine is used to inhibit uptake of [^{3}H]GCV, the concentration of adenine is far greater than its K_m and adenine completely saturates the transporter, resulting in marked inhibition of [^{3}H]GCV uptake.

Corneal permeability values of GCV across a 100-fold concentration range remained constant (**Figure 7**), suggesting that GCV permeates the corneal tissue by passive diffusion and does not involve any carrier-mediated transport process. Moreover, GCV transport across the cornea was not inhibited by 1mM adenine (**Figure 8**). These results thus strengthen our earlier observation that nucleoside/nucleobase transporter expression on the SIRC cell line does not correlate with that on the rabbit cornea.

Cell culture conditions, duration of culture, and degree of differentiation have been reported to influence the level and type of nucleoside/nucleobase transporters expressed.[7,37-39] Del Santo et al[37] reported differential expression of nucleoside transporters in rat liver parenchymal cells and hepatoma cell line. Cell cycle progression and regulation, and degree of differentiation, were shown to be factors responsible for and controlling this differential expression of nucleoside transporters. Pennycooke et al[40] reported that expression patterns of nucleoside transporters vary in normal and tumor tissues. Furthermore, selective loss of nucleoside carrier systems in carcinomas has also been observed.[41] Although SIRC is not a tumor cell line, the process of immortalization of the SIRC cells could have led to changes in the expression pattern. The observed differences in the nucleoside/ nucleobase transporter types expressed on the isolated rabbit cornea and SIRC cell line may thus be evolutionary in nature or could be the result of cell culture conditions. Further studies are necessary to determine whether functional expression of N3-type nucleoside transporters can be induced in the SIRC cell line by changing medium composition, basement matrix, time in culture, and growth factors.

In conclusion, the presence of an "es" type equilibrative nucleoside transporter and a purine-specific nucleobase transporter on the SIRC cell line has been established. However, functional characteristics of these transporters on the SIRC cells differ from those on the isolated rabbit cornea. Transport of nucleosides across the intact rabbit cornea involves a sodium-dependent N3 type nucleoside transporter, whereas carrier-mediated transport is not evident for the purine nucleobases. Such major functional differences between the SIRC cell line and the rabbit cornea with respect to nucleoside/nucleobase transporters, points to the lack of applicability of the SIRC cell line as an in vitro tool for high-throughput screening of nucleoside analogs targeted for topical delivery.

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